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Nicole Rachfall
Vanderbilt University School of Medicine

Alyssa E. Johnson
Vanderbilt University School of Medicine

Sapna Mehta
Vanderbilt University School of Medicine

Jun Song Chen
Vanderbilt University School of Medicine

Kathleen L. Gould
Vanderbilt University School of Medicine

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Cdk1 promotes cytokinesis in fission yeast through activation of the septation initiation network

Nicole Rachfall, Alyssa E. Johnson*, Sapna Mehta†, Jun-Song Chen, and Kathleen L. Gould
Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

ABSTRACT **In *Schizosaccharomyces pombe*, late mitotic events are coordinated with cytokinesis by the septation initiation network (SIN), an essential spindle pole body (SPB)–associated kinase cascade, which controls the formation, maintenance, and constriction of the cytokinetic ring. It is not fully understood how SIN initiation is temporally regulated, but it depends on the activation of the GTPase Spg1, which is inhibited during interphase by the essential bipartite GTPase-activating protein Byr4-Cdc16. Cells are particularly sensitive to the modulation of Byr4, which undergoes cell cycle–dependent phosphorylation presumed to regulate its function. Polo-like kinase, which promotes SIN activation, is partially responsible for Byr4 phosphorylation. Here we show that Byr4 is also controlled by cyclin-dependent kinase (Cdk1)–mediated phosphorylation. A Cdk1 nonphosphorylatable Byr4 phosphomutant displays severe cell division defects, including the formation of elongated, multinucleate cells, failure to maintain the cytokinetic ring, and compromised SPB association of the SIN kinase Cdc7. Our analyses show that Cdk1-mediated phosphoregulation of Byr4 facilitates complete removal of Byr4 from metaphase SPBs in concert with Plo1, revealing an unexpected role for Cdk1 in promoting cytokinesis through activation of the SIN pathway.**

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INTRODUCTION

In many organisms, cytokinesis requires assembly of an actomyosin-based contractile ring (CR), whose constriction results in the physical separation of one mother cell into two genetically identical daughter cells. The fidelity of this process depends on the centrosome in metazoans or the spindle pole body (SPB) in yeast cells (Piel *et al.*, 2001; Magidson *et al.*, 2006). These conserved organelles anchor many regulatory proteins involved in cell cycle control.

A major cytokinesis-regulating pathway anchored at SPBs is the essential septation initiation network (SIN) in the fission yeast *Schizosaccharomyces pombe*. The SIN coordinates nuclear division with cytokinesis from the SPB by directing the formation, maintenance, and constriction of the CR, as well as septum formation (Balasubramanian *et al.*, 1998; Jin *et al.*, 2006; Hachet and Simanis, 2008; reviewed in Gould and Simanis, 1997; Johnson *et al.*, 2012). In addition, the SIN inhibits polarized cell growth during mitosis and is involved in regulating anaphase spindle elongation and positioning the nuclei away from the cell division site in telophase via inhibition of Klp2, a minus end–directed kinesin-14 (Mana-Capelli *et al.*, 2012; Gupta *et al.*, 2013). The SIN is the analogue of the mitotic exit network (MEN) in *Saccharomyces cerevisiae*. Many core components involved in SIN and MEN signaling are conserved in metazoans and share homology and functional similarities to proteins of the Hippo pathway, which controls cell cycle exit (Reddy *et al.*, 2010; reviewed in Halder and Johnson, 2011), as well as cell proliferation and apoptosis, processes frequently deregulated in tumor cells (Yang *et al.*, 2004; Bothos *et al.*, 2005; reviewed in Halder and Johnson, 2011; Avruch *et al.*, 2012; Hergovich, 2012).

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Present addresses: *Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158; †Department of Biology, Indiana University, Bloomington, IN 47405.

Address correspondence to: K. L. Gould (kathy.gould@vanderbilt.edu).

Abbreviations used: Cdk, cyclin-dependent kinase; CR, contractile ring; MBP, maltose-binding protein; MEN, mitotic exit network; SIN, septation initiation network; SPB, spindle pole body.

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The SIN pathway consists of the SPB-binding scaffold complex Cdc11-Sid4 and an associated kinase signaling cascade whose activity is controlled by the small Ras-superfamily GTPase Spg1 and its bipartite GTPase-activating protein (GAP) Byr4-Cdc16 (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997; Furge *et al.*, 1998; Chang and Gould, 2000; Krapp *et al.*, 2001; Tomlin *et al.*, 2002; Morrell *et al.*, 2004). Spg1 resides at SPBs throughout the cell cycle (Schmidt *et al.*, 1997; Sohrmann *et al.*, 1998). During interphase, Spg1 is kept in an inactive, GDP-bound state by its association with Byr4-Cdc16, which prevents premature SIN signaling and onset of cytokinesis (Furge *et al.*, 1998). Upon mitotic entry, Byr4-Cdc16 dissociates from the SPB (Cerutti and Simanis, 1999; Li *et al.*, 2000), allowing Spg1 to switch to its active GTP-bound state and initiate SIN signaling through its effector kinase Cdc7 and subsequent activation of the downstream kinase modules Sid1-Cdc14 and Sid2-Mob1 (Sparks *et al.*, 1999; Guertin *et al.*, 2000). Mutations in genes encoding proteins involved in the SIN pathway are lethal and cause severe cytokinetic defects. Loss-of-function mutations in SIN activators (*spg1*, *cdc7*, *sid1*, *cdc14*, *sid2*, *mob1*) lead to the development of elongated cells with multiple nuclei (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; Schmidt *et al.*, 1997; Balasubramanian *et al.*, 1998), whereas mutations in SIN inhibitors (*byr4*, *cdc16*) result in the formation of multiple septa in one yeast cell without cell cleavage (Minet *et al.*, 1979; Song *et al.*, 1996).

The initiation of SIN signaling is spatially controlled. In prometaphase, just after SPB separation, the SIN inhibitor Byr4-Cdc16 is associated with both SPBs but then dissociates from both poles in metaphase, allowing for symmetric binding of the initiator kinase Cdc7 to Spg1-GTP (Sohrmann *et al.*, 1998; Cerutti and Simanis, 1999). During anaphase B, Byr4-Cdc16 returns and terminates SIN signaling on the old pole, resulting in asymmetric SIN activation on the new, SIN activator-associated SPB (Sohrmann *et al.*, 1998; Cerutti and Simanis, 1999; Li *et al.*, 2000; Grallert *et al.*, 2004). A recent computational study shows that the minimal regulatory unit required to establish SIN asymmetry consists of the antagonistic interaction between the entity of SIN activators on one side and the inhibitory GAP complex Byr4-Cdc16 on the other (Bajpai *et al.*, 2013). This study illustrates the particular significance of Byr4-Cdc16 in asymmetric SIN regulation, which contributes to silencing the SIN after cytokinesis is complete (Grallert *et al.*, 2004; Garcia-Cortes and McCollum, 2009; Singh *et al.*, 2011; Feoktistova *et al.*, 2012; Bajpai *et al.*, 2013). Thus SIN initiation is under not only strict spatial, but also distinct temporal control, ensuring that cytokinesis does not occur until chromosome segregation is complete. However, the trigger that determines SIN activation or inhibition and the distinct targets of such regulation within the SIN pathway have not been fully elucidated.

Because Byr4 is a pivotal player in controlling SIN activity, it is an attractive regulatory target (Moriya *et al.*, 2011; Bajpai *et al.*, 2013). Whereas highly increased levels of its GAP partner Cdc16 do not affect cytokinesis or cell cycle progression (Krapp *et al.*, 2008; Moriya *et al.*, 2011), even slight overexpression of *byr4* is lethal, resulting in the inhibition of cytokinesis and the development of multinucleate cells (Song *et al.*, 1996). In addition, Byr4 is a phosphoprotein, and the absence of Byr4-Cdc16 from both SPBs in metaphase and their asymmetric localization in anaphase coincides with hyperphosphorylation of Byr4 at the metaphase–anaphase transition (Krapp *et al.*, 2008) and the activity peaks of Plo1 and cyclin-dependent kinase 1 (Cdk1; Grallert *et al.*, 2013), which are major regulators of cytokinesis.

Plo1 is recognized as the most upstream activator of the SIN pathway and associates with mitotic SPBs partly through an interaction with the SIN scaffold protein Sid4 (Chang and Gould, 2000; Krapp *et al.*, 2001; Tanaka *et al.*, 2001; Mulvihill and Hyams, 2002; MacIver *et al.*, 2003; Morrell *et al.*, 2004; Grallert *et al.*, 2013). In agreement with a function for Plo1 in SIN activation, the Polo-like kinase Cdc5 phosphorylates the Byr4 homologue Bfa1 in *S. cerevisiae* (Hu *et al.*, 2001; Kim *et al.*, 2012). Phosphoregulation by Cdc5 inhibits Bfa1 SPB-binding and Bfa1-Bub2 GAP activity, resulting in the activation of the mitotic exit network (MEN; Hu *et al.*, 2001; Geymonat *et al.*, 2002; Kim *et al.*, 2008, 2012). In *S. pombe*, Plo1 phosphorylates Byr4 in vitro and contributes to Byr4 phosphorylation in vivo (Johnson and Gould, 2011). In analogy with the situation in *S. cerevisiae*, this likely contributes to Byr4 inhibition.

Cdk1, on the other hand, is generally regarded as global inhibitor of cytokinesis, as low Cdk1 activity is a hallmark of mitotic exit (reviewed in Bohnert and Gould, 2011). Although it promotes mitotic commitment, cytokinesis is not initiated until late mitosis, when Cdk1 activity declines (He *et al.*, 1997; Guertin *et al.*, 2000; Chang *et al.*, 2001; Wolf *et al.*, 2007; Dischinger *et al.*, 2008). Cdk1 inactivation stimulates cytokinesis in mammalian cells without preceding chromosome segregation (Niiya *et al.*, 2005), and in *S. pombe* the association of the SIN kinase module Sid1-Cdc14 to the SPB is inhibited by Cdk1 (Guertin *et al.*, 2000). Thus Cdk1 is believed to inhibit the SIN, but prevention of Cdk1-mediated phosphorylation of Cdc11, the only identified Cdk1 target within the SIN, does not have a significant effect on cytokinesis, suggesting the existence of other Cdk1-targeted SIN proteins (Morrell *et al.*, 2004; Chen *et al.*, 2013).

Here we find that the SIN inhibitor Byr4 is targeted by Cdk1. In spite of an anticipated inhibitory function of this event on cytokinesis, our study provides evidence for an activating role of Cdk1 in cytokinesis via phosphoregulation of Byr4 in metaphase. We demonstrate that Cdk1 and Plo1 kinase act together in the removal of Byr4 from mitotic SPBs to allow for the initiation of SIN signaling and accuracy of cytokinesis.

RESULTS

Byr4 is a Cdk1 substrate

Because the absence of Byr4 from SPBs correlates temporally with a peak in Cdk1 activity and the absence of Plo1 activity only partially eliminates Byr4 phosphorylation (Johnson and Gould, 2011), we sought to investigate whether Cdk1 might be involved in Byr4 phosphoregulation. Recombinant Byr4 was subjected to an in vitro kinase assay with Cdk1 purified from insect cells (Cdc2-Cdc13). We found that Byr4 is indeed an excellent Cdk1 target in vitro (Figure 1A). Phospho–tryptic peptide maps generated from in vitro–phosphorylated Byr4 and in vivo metabolically labeled Byr4 (Supplemental Materials and Methods) show significant overlap of phosphorylated tryptic peptides, indicating that Byr4 is a likely Cdk1 target in vivo (Supplemental Figure S1).

The amino acid sequence of Byr4 harbors 11 Cdk1 consensus motifs (S/T*-P, where the asterisk indicates the site of phosphorylation). To determine which of these sites are phosphorylated by Cdk1, we tagged Byr4 with a histidine-biotin-histidine (HBH) tag and purified Byr4-HBH under fully denatured conditions from mitotically arrested cells (when the highest levels of Byr4 phosphorylation are observed; Krapp *et al.*, 2008). Purified Byr4-HBH was subjected to two-dimensional liquid chromatography tandem mass spectrometry (2D-LC MS/MS) analysis. Phosphorylation of residues S248, S326, T429, S499, T502, and S533, matching the Cdk1 consensus motif, was detected confidently (Supplemental Table S2). In addition to these six in vivo–identified phosphorylation sites, T412, which lies on a tryptic peptide that was not covered by the in vivo analyses, was identified as a phosphorylated residue by 2D-LC MS/MS analysis of in vitro Cdk1-phosphorylated recombinant Byr4 protein (Supplemental Table S2). Three of the in vivo identified

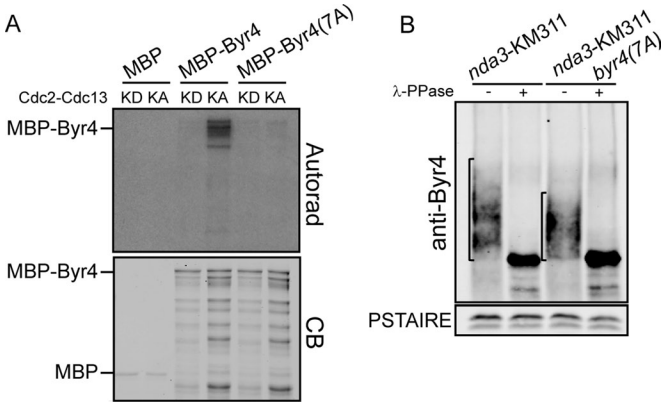

FIGURE 1: Byr4 is a Cdk1 substrate. (A) Top, recombinant MBP-purified proteins were treated in vitro with kinase-active (KA) or kinase-dead (KD) Cdk1 complex (Cdc2-Cdc13) and subjected to SDS–PAGE, followed by autoradiography. Bottom, Coomassie blue (CB) gel of purified recombinant MBP, MBP-Byr4, and MBP-Byr4(7A) proteins. (B) Byr4 and Byr4(7A) were immunoprecipitated from mitotically arrested cells of the indicated backgrounds. Gel shifts were visualized by anti-Byr4 immunoblotting of Phos-tag gels (2 μM) and incubation of immunoprecipitates with (+) or without (–) λ-phosphatase (λ-PPase). Anti-PSTAIRE antibody served as loading control for lysates used in immunoprecipitations.

residues—serines 326, 499 and 533—were identified previously to be phosphorylated in Byr4 via global in vivo phosphoproteomics studies (Wilson-Grady *et al.*, 2008; Beltrao *et al.*, 2009).

All seven serine and threonine residues identified as Cdk1 target sites were mutated to alanine, and the resulting Byr4(7A) recombinant protein was subjected to a Cdk1 in vitro kinase assay to determine whether Cdk1-mediated Byr4 phosphorylation was abolished. Whereas Cdk1 phosphorylates maltose-binding protein (MBP)–Byr4, no signal was detectable when MBP-Byr4(7A) was incubated with Cdk1, confirming that Cdk1-mediated phosphorylation of Byr4 is abolished in this mutant (Figure 1A). The wild-type allele of *byr4* was then replaced with the *byr4(7A)* mutant allele. We detected a reduced mobility shift for immunoprecipitated Byr4(7A) in comparison to wild-type Byr4 in mitotically arrested cells (Figure 1B). Both bands were completely collapsed upon treatment with λ-phosphatase, indicating that the reduced mobility of Byr4(7A) relative to Byr4 is due to a loss of phosphorylation. The remaining phosphorylation observed for the Byr4(7A) phosphomutant is at least partially due to Plo1 kinase activity, which contributes considerably to Byr4 phosphorylation (Johnson and Gould, 2011). Taken together, these data identify Byr4 as a bona fide Cdk1 substrate and demonstrate that Cdk1 is responsible for a significant amount of Byr4 phosphorylation in vivo.

Byr4(7A) is a hyperactive inhibitor of the SIN pathway

We next analyzed the phenotypes associated with endogenous expression of the *byr4(7A)* allele. Whereas wild-type cells generally showed a normal distribution of nuclei and septa, cytokinetic defects observed for the *byr4(7A)* mutant included the formation of binucleate cells, in which the two nuclei inappropriately clustered in the cell middle ("kissing nuclei," a) and elongated, multinucleate cells either with (c) or without (b) septum (Figure 2A and

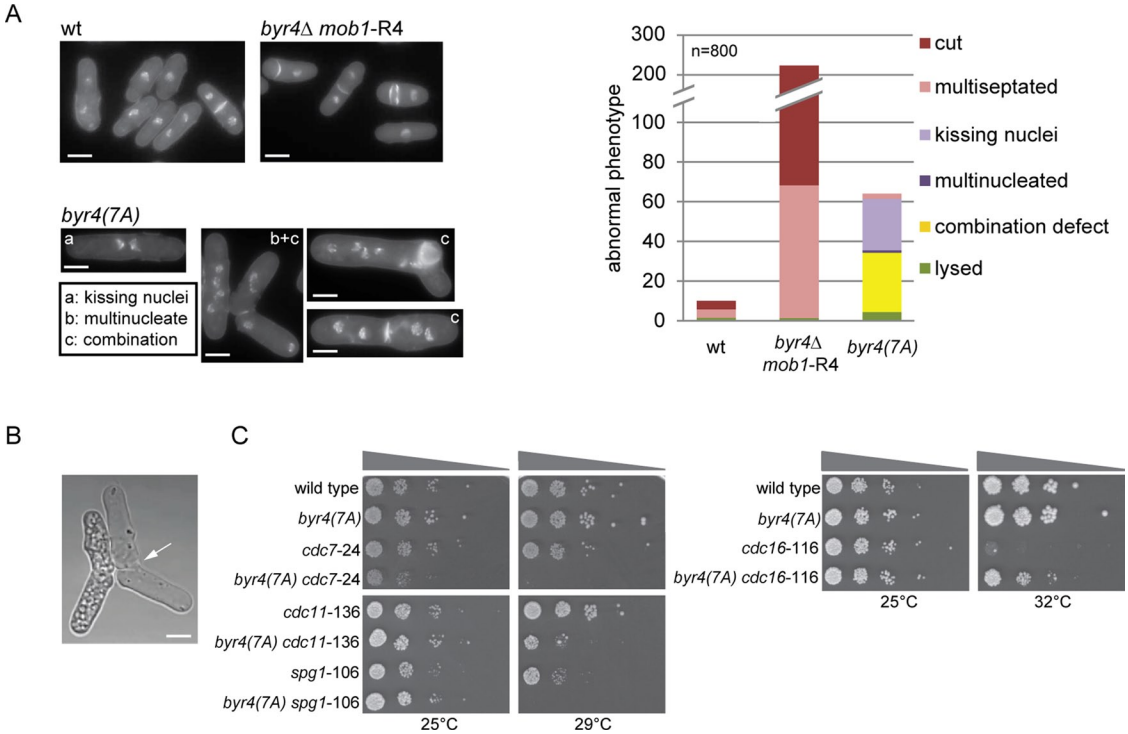

FIGURE 2: Preventing Cdk1 phosphorylation of Byr4 causes cytokinetic defects. (A) Wild type, *byr4(7A)*, and a strain deleted for *byr4* (*byr4*Δ *mob1*-R4) were grown to early-mid log phase at 25°C. Cells were fixed and stained with DAPI and methyl blue. Representative images. Scale bar, 3 μm. Cells displaying an abnormal septation phenotype are quantified ($n = 800$). (B) Representative image of time-lapse microscopy (20-min intervals) of *byr4(7A)* cells. Arrow highlights protrusion at division site. Scale bar, 5 μm. (C) Cells of the indicated genotypes were spotted on YE media in 10-fold serial dilutions, and plates were imaged after incubation for 3 d at the indicated temperatures.

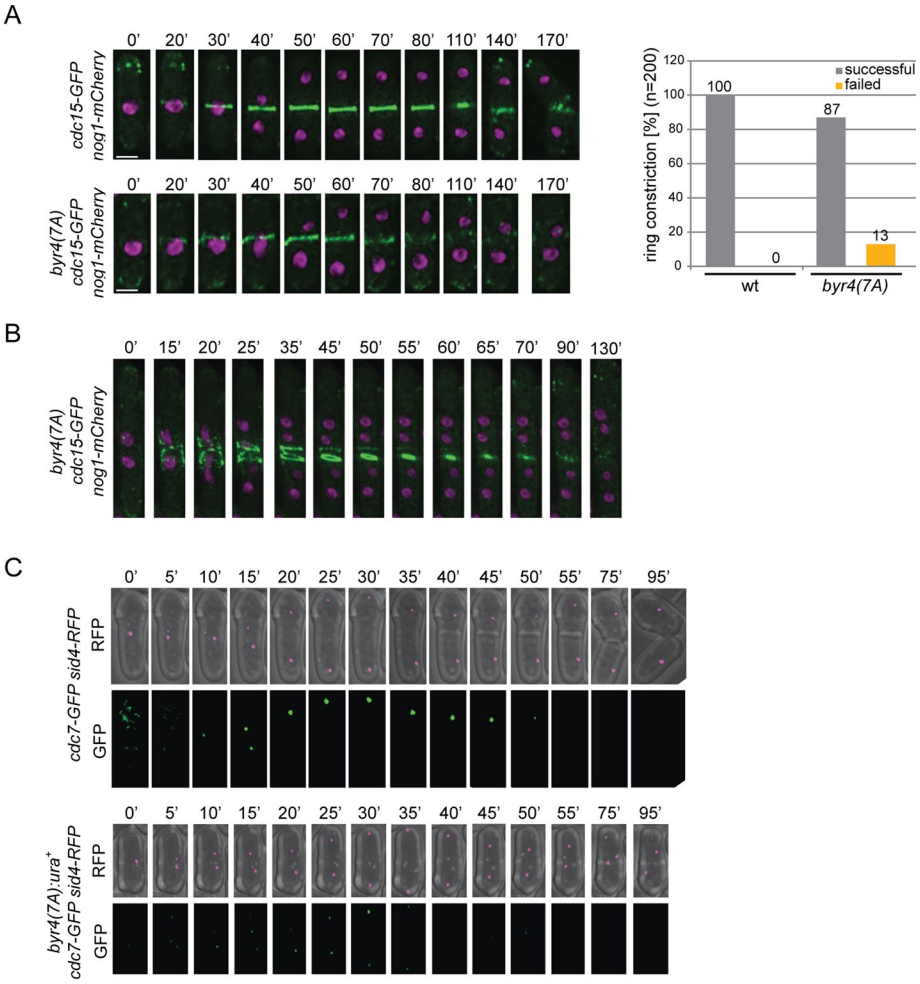

FIGURE 3: Preventing Cdk1-mediated phosphorylation of Byr4 compromises SIN signaling. (A) Representative montages of time-lapse microscopy (5-min intervals) in the indicated genetic backgrounds (Supplemental Videos S2 and S3). Cdc15-GFP was used as ring marker and Nog1-mCherry as nucleolar marker. Scale bar, 3 μm. (B) Representative montages of time-lapse microscopy (5-min intervals) illustrating cytokinesis in a binucleate cell of the *byr4(7A)* background (Supplemental Video S4). Scale bar, 3 μm. (C) The dynamics of GFP-tagged Cdc7 SPB localization was monitored by time-lapse microscopy (5-min intervals) in the indicated genetic backgrounds (Supplemental Videos S5 and S6). The time point just before spindle pole body separation was set to 0 min. Sid4-RFP served as a constitutive SPB marker. Representative montages. Scale bar, 3 μm.

Supplemental Figure S2). Both of these defects are associated with compromised SIN signaling and cytokinetic failure (Roberts-Galbraith *et al.*, 2009). These phenotypes are in stark contrast to the phenotypes associated with a *byr4*-null mutant (viable only in the *mob1*-R4 background), which is characterized by an accumulation of cells with multiple septa and "cut" cells, corresponding to multiple rounds of cytokinesis and SIN hyperactivation (Song *et al.*, 1996; Figure 2A and Supplemental Figure S2).

In addition to these indicators of impaired cytokinesis in the *byr4(7A)* mutant, cell lysing during cell division, as well as cellular bulging and protrusions at the division site, was observed by time-lapse microscopy of *byr4(7A)* cells (Figure 2B, Supplemental Figure S3, and Supplemental Video S1). These data suggest compromised cell wall integrity in the *byr4(7A)* strain, a phenotype previously linked to reduced SIN activity (Cortes *et al.*, 2002). These combined indications of compromised SIN signaling in the *bry4(7A)* phosphomutant strain are further supported by negative genetic interactions displayed by the *byr4(7A)* strain and temperature-sensitive alleles of the SIN activators Cdc7, Cdc11, and Spg1 at the restrictive temperature (Figure 2B). Conversely, *byr4(7A)* partially rescued the growth defect of a strain expressing the temperature-sensitive allele (*cdc16*-116) of the SIN inhibitor Cdc16 at the restrictive temperature. Taken together, these data indicate that abolishing Cdk1-mediated phosphorylation of Byr4 produces a gain-of-function allele, rendering Byr4(7A) a hyperactive SIN inhibitor.

Cdk1-mediated phosphorylation of Byr4 promotes SIN function

We next asked whether the key functions of the SIN pathway—the formation, maintenance, and constriction of the CR, were affected in the *byr4(7A)* strain. We followed ring dynamics throughout the cell cycle by time-lapse imaging using green fluorescent protein (GFP)–tagged Cdc15 as a marker (Fankhauser *et al.*, 1995). Both wild-type and *byr4(7A)* mutant cells were capable of initial CR formation, but with 13%, a significant fraction of the *byr4(7A)* phosphomutant cells did not maintain the CR in order to support successful ring constriction and cytokinesis (Figure 3A and Supplemental Videos S2 and S3). Instead, the contractile ring disintegrated soon after it had been established, resulting in cytokinetic failure and the formation of binucleate cells displaying "kissing nuclei" in the cell middle (Figure 3A, bottom, and Supplemental Video S3). These binucleate cells were not predestined to show the same cytokinetic defects in the next round of cell division but were able to undergo successful cytokinesis. In this process, two CR structures were formed per binucleate cell, but only one cell division event took place, producing two binucleate daughter cells (Figure 3B and Supplemental Video S4; Okazaki and Niwa, 2008).

To further ascertain whether cytokinesis in *byr4(7A)* cells is compromised as a consequence of improper SIN signaling, we monitored the SPB localization of the initiator SIN kinase Cdc7, which accumulates at the SPB with active SIN signaling and is therefore often used as an indicator for SIN activation (e.g., Garcia-Cortes and McCollum, 2009; Figure 3C and Supplemental Videos S5 and S6). In wild-type cells, Cdc7-GFP localized symmetrically to both SPBs in metaphase until 10 min after SPB separation (time point 15 min) and then switched to asymmetric SBP association in anaphase, a situation that was maintained until cytokinesis was complete (Figure 3C, top, and Supplemental Video S5). In cells of the *byr4(7A)* background, the symmetric localization of Cdc7-GFP persisted further into anaphase, and the overall signal strength seemed to be reduced. In cells that failed cytokinesis, symmetric Cdc7 localization was significantly extended up to 30 min after SPB separation (Figure 3C, bottom, and Supplemental Video S6). During this time period, Cdc7-GFP signal intensities often differed between both poles, and the preference for either pole seemed to switch

back and forth (10- to 30-min time points), which was never observed in wild-type cells. The absence of Cdc7 asymmetry in *byr4(7A)* cells was followed by a premature loss of Cdc7 signal from both SPBs and cytokinetic failure (Figure 3C, bottom; compare 40-min time points; and Supplemental Video S6). This is in striking contrast to the strong asymmetric Cdc7-GFP signal in wild-type cells, which persisted throughout the initiation of septum formation (Figure 3C, top, and Supplemental Video S5). The reduced SPB association and lack of Cdc7 asymmetry in *byr4(7A)* cells is likely responsible for insufficient SIN function and subsequent failure in cytokinesis and septum formation. These experiments establish that Byr4 phosphorylation by Cdk1 contributes to full SIN activation and proper execution of cytokinesis.

Cdk1 and Polo kinase regulate Byr4 SPB association during the metaphase–anaphase transition

Next we investigated the cause of diminished Cdc7 SPB localization in the *byr4(7A)* phosphomutant. Because Byr4 and Cdc7 both bind to the GTPase Spg1 at the SPB (Furge *et al.*, 1998; Sohrmann *et al.*, 1998; Mehta and Gould, 2006), we hypothesized that Byr4(7A) might antagonize Cdc7 SPB association. As expected, no difference in Byr4 SPB localization between wild type and the Cdk1 nonphosphorylatable *byr4(7A)* mutant was detected in anaphase when Cdk1 is inactive (Figure 4A). However, during metaphase, when Byr4 is lost from both SPBs in wild-type cells, Byr4(7A) remained associated with one or both SPBs in most cells, indicating that Cdk1 has a significant effect on the removal of Byr4 from metaphase SPBs (Figure 4A).

Because the lack of Byr4 phosphorylation by Cdk1 did not lead to a constitutive association of Byr4(7A) with both SPBs, but instead most cells showed asymmetric Byr4(7A) SPB localization, we hypothesized that complete removal of Byr4 from metaphase SPBs might require a second mechanism in addition to direct Cdk1 phosphorylation. Another kinase that is active during metaphase and known to phosphorylate Byr4 is Plo1 (Johnson and Gould, 2011). Thus we reasoned that Plo1 might also contribute to the removal of Byr4 from metaphase SPBs. This possibility is supported by a negative genetic interaction between *byr4(7A)* and the temperature-sensitive *plo1*-24c allele at both semipermissive (32°C) and restrictive (36°C) temperatures (Figure 4B). To test our hypothesis that Cdk1 and Plo1 contribute to Byr4 SPB removal, we determined the SPB distribution of Byr4 and Byr4(7A) in the *plo1*-24c background at permissive (25°C) and semipermissive (32°C) temperatures (Figure 4A). The *plo1*-24c mutation is hypomorphic and primarily defective in CR structure and location (Bahler *et al.*, 1998). At its semipermissive temperature, Plo1 function is reduced but not to the extent of preventing spindle formation as in a *plo1*Δ strain (Ohkura *et al.*, 1995), which enabled us to monitor Byr4 localization not only at metaphase but also in anaphase.

When the *plo1*-24c strain was grown at the permissive temperature, the Byr4 and Byr4(7A) localization corresponded to the

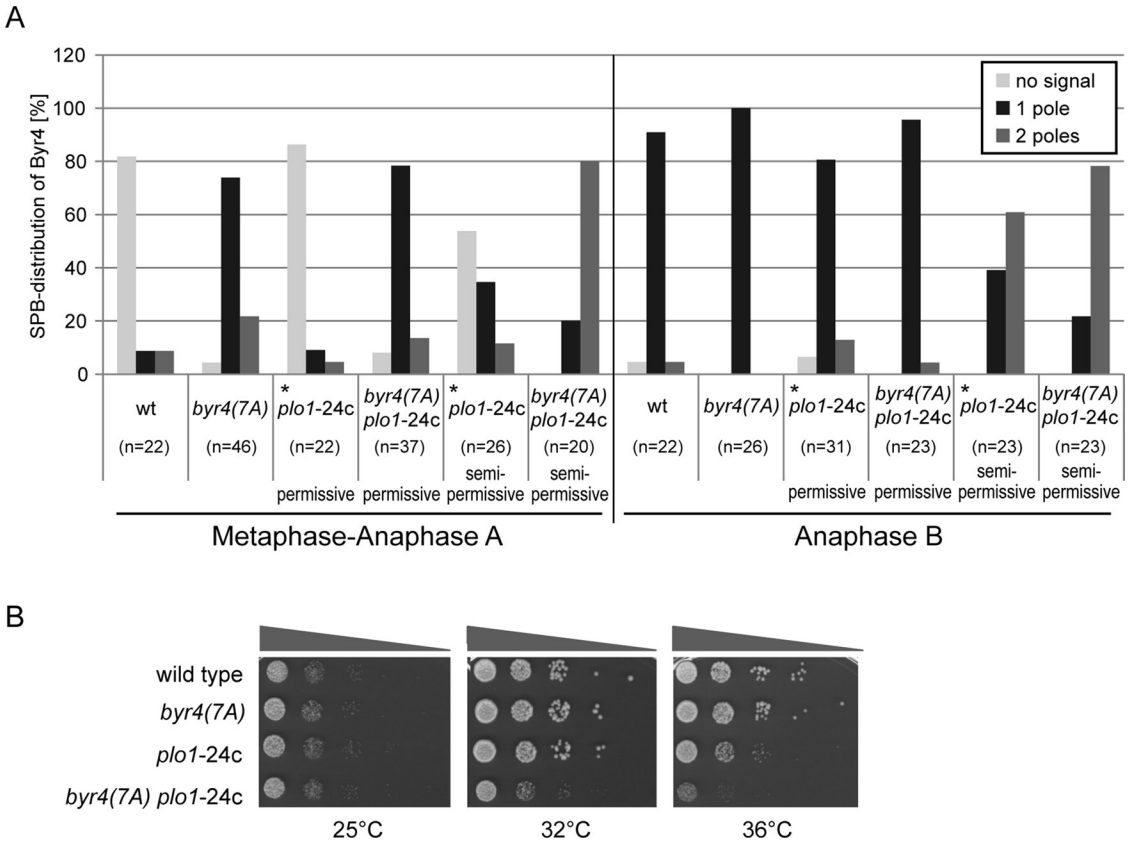

FIGURE 4: Cdk1 and Plo1 independently and additively mediate Byr4 removal from mitotic SPBs. (A) Byr4 localization was detected by indirect immunofluorescence or 3xGFP-tag (indicated by *, *plo1*-24c *byr4*-3xGFP). Wild-type and *byr4(7A)* cells were grown at 32°C. Strains containing the temperature-sensitive *plo1*-24c allele were grown at the permissive temperature (25°C) and, where indicated, shifted to the semipermissive temperature (32°C) for 3 h before fixation. The distribution of Byr4 on mitotic SPBs in the respective strains at the indicated temperatures is illustrated. (B) Serial 10-fold dilutions of cells of the indicated genotypes were plated on YE media. Plates were imaged after incubation for 3 d at the indicated temperatures.

localization observed in wild-type and *byr4(7A)* mutant cells, respectively, throughout mitosis (Figure 4A). During anaphase at the semipermissive temperature, Byr4 predominantly localized to both anaphase SPBs in *plo1*-24c cells as opposed to an asymmetric localization observed for *plo1* wild-type cells (wild-type and *bry4(7A)*; Figure 4A, Anaphase B). This effect is slightly exacerbated when, in addition, Byr4 is nonphosphorylatable by Cdk1 (Figure 4A, Anaphase B), potentially due to the function of Cdk1 in the initial removal of Byr4 from metaphase SPBs (Figure 4A, Metaphase-Anaphase A).

In metaphase, a reduction of Plo1 activity at semipermissive temperature led to increased accumulation of Byr4 at metaphase SPBs, whereas the effects are not as strong as observed for the byr4(7A) mutant strain (Figure 4A, Metaphase-Anaphase A). This could imply a stronger role for Cdk1 than Plo1 in Byr4 phosphoregulation during metaphase, but it might also result from the incomplete inhibition of Plo1 at the semipermissive temperature. In the *byr4(7A) plo1*-24c double mutant strain grown at the semipermissive temperature, removal of Byr4 from metaphase SPBs was further impeded compared with the *byr4(7A)* or *plo1*-24c single mutants, resulting in Byr4(7A) localizing to both metaphase SPBs in 80% of cells.

In conclusion, these data indicate that the initial removal of Byr4 from both SPBs during metaphase is ensured by the combined activities of Cdk1 and Plo1. Whereas Cdk1 phosphoregulation of Byr4 is limited to metaphase, Plo1 continuously acts in Byr4 SPB removal throughout anaphase.

Cdk1-mediated phosphorylation of Byr4 is sufficient to drive cells into cytokinesis even when Polo kinase activity is compromised

After finding that Cdk1-mediated phosphorylation of Byr4 functions in the regulation of Byr4 SPB localization in metaphase, we were interested in evaluating the importance of this regulation for cytokinetic fidelity. This analysis was complicated by our finding that Plo1 and Cdk1 are both involved in the phosphoregulation and SPB localization of Byr4. To isolate the significance of Cdk1-mediated Byr4 phosphorylation, we assessed the ability of cells to undergo cytokinesis in a *plo1*-24c temperature-sensitive background. Cells were synchronized in S phase and shifted to the restrictive temperature of 36°C. Our data show that cells enter cytokinesis and form septa when Plo1 function is compromised by the *plo1*-24c allele but exhibit significant cytokinetic defects (Figure 5) as observed previously (Bahler *et al.*, 1998). More than 60% of *plo1*-24c cells formed multiple septa or showed septum formation in mononucleate cells ("cut" phenotype). In addition, the majority of septa were disorganized and misplaced, forming networks of septal material and occasionally running along the long axis of the cell, in accord with previous observations of the *plo1*-24c allele (Bahler *et al.*, 1998). In contrast, *byr4(7A) plo1*-24c double mutant cells displayed a much more complete block to cytokinesis, as evidenced by a lack of septum formation (Figure 5). In addition, almost half of *byr4(7A) plo1*-24c cells showed "kissing nuclei" in their cell middle or multinucleation, which indicates previous and repeated cytokinetic failure, respectively. These phenotypes were virtually absent in the *plo1*-24c mutant (Figure 5). In conclusion, these data indicate that Cdk1-mediated phosphorylation and removal of Byr4 from metaphase SPBs plays a significant role in the initiation of cytokinesis, as it can drive entry into cytokinesis even when Plo1 function is compromised.

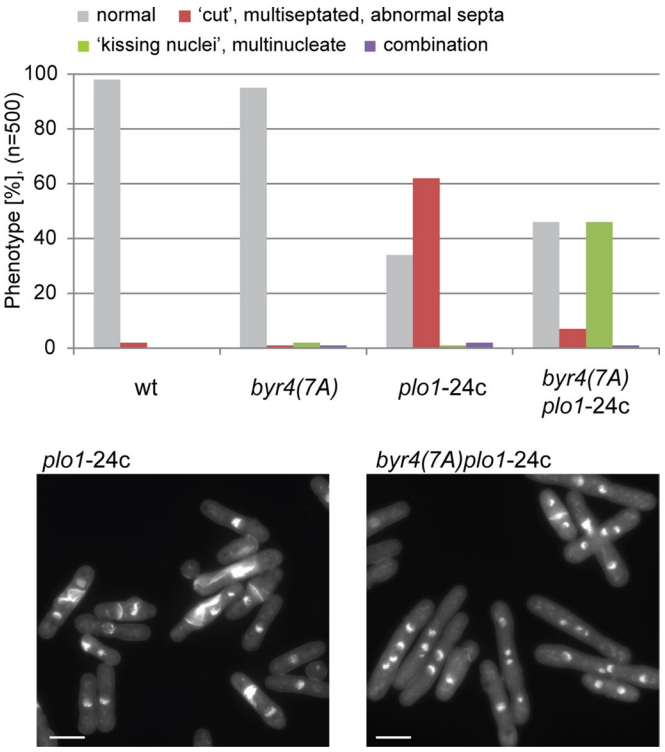

FIGURE 5: Byr4 phosphorylation by Cdk1 is important for cytokinesis. Cells of the indicated genetic backgrounds were grown at the permissive temperature (25°C) for *plo1*-24c and arrested in S phase (HU) before a shift to the restrictive temperature (36°C) for 3 h before fixation. Respective phenotypes were assessed by septa and nuclei staining and quantified ($n = 500$). Representative micrographs of the *plo1*-24c and *plo1*-24c *byr4(7A)* strains. Scale bar, 5 μm.

DISCUSSION

Even though the SIN inhibitor Byr4 has been denoted as a phosphoprotein for a significant length of time (Song *et al.*, 1996; Krapp *et al.*, 2008), the effect of its phosphorylation has remained largely elusive. In this study, we find that the loss of Cdk1-mediated phosphorylation of Byr4 compromises SIN function, resulting in failure to maintain the CR and a reduced and misdirected SPB association of the SIN initiator kinase Cdc7. These effects are linked to a role for Cdk1 in removing Byr4 from metaphase SPBs and indicate a novel function for Cdk1 in priming the SIN pathway for cytokinesis (Figure 6), opposed to its hitherto-assumed sole function in the

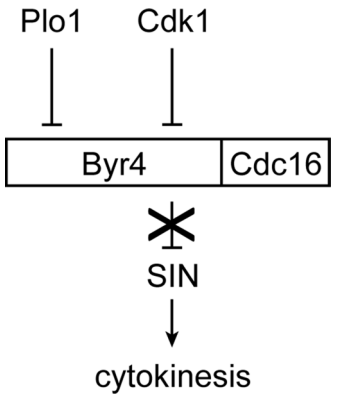

FIGURE 6: Schematic of Byr4 phosphoregulation. During mitosis, Cdk1 and Plo1 phosphorylate Byr4 independently, leading to the removal of Byr4 from SPBs and the inhibition of the GAP Byr4-Cdc16. Consequently, SIN activation and cytokinesis are induced.

inhibition of cytokinesis. In addition, our findings reveal molecular mechanisms that can lead to the establishment of asymmetry within a signaling pathway.

Our results illustrate that Cdk1 and Plo1 kinase cooperate in Byr4 removal from mitotic SPBs (Figure 6). Even though cyclin-dependent kinases are able to prime substrates for Polo kinases (Elia *et al.*, 2003; Almonacid *et al.*, 2011), Cdk1 and Plo1 seem to target Byr4 independently. This is supported by the synthetic growth defect observed in a *byr4(7A) plo1*-24c double mutant. In addition, Byr4(7A) retains a significant level of in vivo phosphorylation, likely attributed to Plo1 kinase (Johnson and Gould, 2011). Although we cannot exclude that Byr4-independent functions of Cdk1 facilitate Byr4 phosphoregulation by Plo1, similar to the dependence of Plo1 G2-SPB localization on Cdk1 activity (Grallert *et al.*, 2013), our data indicate that Byr4 phosphoregulation by Cdk1 and Plo1 represents two independent mechanisms mediating Byr4 removal from mitotic SPBs in order to allow efficient Cdc7 SPB localization and the establishment of SIN asymmetry, required for proper initiation of cytokinesis (Figure 6). Plo1 and Cdk1 might also act cooperatively in the promotion of the MEN in budding yeast through Bfa1 regulation, as Cdc5 activates the MEN through Bfa1 phosphorylation and the Cdk1-antagonizing phosphatase Cdc14 dephosphorylates Bfa1, thereby reactivating Bfa1-Bub2 at mitotic exit (Pereira *et al.*, 2002; Geymonat *et al.*, 2003).

A significant fraction of *byr4(7A)* cells showed reduced SIN activity and cytokinetic failure, whereas other *byr4(7A)* cells underwent seemingly normal cell division (Figure 3). Even though cells have the same genetic background and grow under identical environmental conditions, the displayed phenotypes can be variable (Munsky *et al.*, 2012). In the *byr4(7A)* background, this might be due to partial redundancy in the phosphoregulation of Byr4 by Cdk1 and Plo1, equally affecting Byr4 SPB localization during metaphase (Figure 4). For example, we observed that Byr4(7A) was not retained on both metaphase SPBs until Plo1 function was also compromised. Thus SIN signaling is not completely ablated in a *byr4(7A)* strain, but instead an intermediate situation emerges that reduces, but not entirely prevents, Cdc7 SPB association in metaphase, when Cdk1-mediated Byr4 phosphorylation occurs (Figures 3C and 4). Previous work showed that reduced levels of Cdc7 can be sufficient for SIN initiation (Magidson *et al.*, 2006), which is supported by our observation of reduced Cdc7 levels at some *byr4(7A)* SPBs that were able to successfully undergo cytokinesis (unpublished data). In addition, low levels of Byr4 are found at metaphase SPBs during the symmetric SPB association of Cdc7 (Simanis, 2003; Lattmann *et al.*, 2009; Bajpai *et al.*, 2013). These findings suggest that the ratio of Byr4 and Cdc7 present at metaphase SPBs might determine the cytokinetic fate of a cell and that SIN pathway initiation requires Cdc7 SPB levels reaching a certain threshold. Because Byr4 is removed from SPBs by Cdk1 as well as Plo1 kinase, Plo1-mediated phosphorylation of Byr4(7A) might be sufficient to allow for a level of Cdc7 SPB association that can mediate SIN activation and cytokinetic initiation in some *byr4(7A)* cells, whereas Byr4(7A) removal from metaphase SPBs in other cells might be inhibited to a degree that does not allow for sufficient Cdc7 SPB association due to a lack of Cdk1-mediated contribution to Byr4 phosphorylation. Hence we observed that Byr4(7A) in some cells was retained at one SBP and in others at both.

Our data further suggest that establishing SIN asymmetry in anaphase heavily relies on Plo1 function, as Byr4 asymmetry is largely lost in the *plo1*-24c strain even at the semipermissive temperature. Similarly, Bfa1 phosphomutants in *S. cerevisiae* that are not efficiently phosphorylated by Cdc5 lose their asymmetric SPB localization and instead localize symmetrically to both SPBs, resulting in delayed mitotic exit (Kim *et al.*, 2012). Accordingly, hyperphosphorylation of Bfa1 results in a reduced ability of Bfa1 to interact with Tem1 at SPBs (Hu *et al.*, 2001). These combined findings imply that Plo1 activity toward Byr4 is likely limited to the new SPB and establish Plo1 as the currently most upstream regulator of SIN asymmetry. As such, the signals governing Plo1 asymmetric activity will be an important topic to address in the future.

In addition, SIN asymmetry in anaphase seems to be promoted by Cdk1-mediated phosphorylation of Byr4 in metaphase. *byr4(7A)* cells that failed cytokinesis showed a reduced association of Cdc7 to metaphase SPBs, followed by the compromised establishment of asymmetric Cdc7 SPB localization, suggesting that Cdk1 phosphorylation of Byr4 in metaphase sets the stage for proper cell cycle progression (Figure 3C). These observations support the notion that the establishment of asymmetry is required for complete SIN activation (Bajpai *et al.*, 2013). To follow up on this, we tested a possible genetic interaction of the *byr4(7A)* allele and deletions of components of the SIP, a phosphatase complex required for SIN asymmetry (Singh *et al.*, 2011). Indeed, the *byr4(7A) csc3*Δ strain showed a moderate synthetic interaction at lower temperatures (Supplemental Figure S4). Taken together, these data suggest that SIN asymmetry is supported by Cdk1-mediated Byr4 phosphorylation and stable symmetric loading of Cdc7 to both SPBs in metaphase. To break metaphase symmetry and allow for progression of SIN signaling by asymmetric SPB association of Sid1-Cdc14 in anaphase, Cdk1 needs to be inactivated in the metaphase–anaphase transition (Guertin *et al.*, 2000). On the basis of these combined observations, we speculate that continuous Cdk1 activity would inhibit the return of Byr4-Cdc16 to the old SPB in anaphase, an event that is pivotal in establishing SIN asymmetry, thereby preventing mitotic progression. This is analogous to the requirement of Bfa1-Bub2 asymmetric SPB association in budding yeast for mitotic exit (Fraschini *et al.*, 2006; Kim *et al.*, 2012) and in agreement with the observation of failed mitotic exit when Cdk1 inactivation is prevented (Yamano *et al.*, 1996).

In conclusion, our work suggests that Cdk1 phosphoregulation of Byr4 creates an environment permissive for cytokinesis. The removal of Byr4 from metaphase SPBs triggers an initial step in SIN activation. In anaphase, declining Cdk1 activity results in the return of Byr4 to the old SPB while Plo1 keeps Byr4 off the new pole, which in combination allows for SIN signaling to proceed asymmetrically. Thus, although the SIN pathway is not fully activated until anaphase, events during metaphase are required for setting up SIN signaling events. Although our study establishes Byr4 as a significant substrate for Cdk1 in cytokinetic regulation, it alone cannot explain the strong inhibition of cytokinesis when Cdk1 activity remains high. It will be interesting in future studies to further elucidate whether Cdk1 phosphoregulation promotes cytokinesis through additional avenues and whether this previously unrecognized mechanism is conserved in other organisms.

MATERIALS AND METHODS

Yeast strains and growth conditions

S. pombe strains (Supplemental Table S1) used in this study were grown in yeast extract (YE) or Edinburgh minimal medium supplemented with appropriate amino acids (Moreno *et al.*, 1991). For *nda3*-KM311 arrests, cultures were grown overnight at 32°C until mid log phase and shifted to 18°C for 5.5 h before harvesting. Cell cycle progression was observed by synchronizing cells in S phase using hydroxyurea (HU) at a final concentration of 12 mM. HU was washed out after 5 h of growth at 25°C (permissive temperature), and the cultures were shifted to 36°C (restrictive temperature) for 3 h before fixation.

Strain KGY14957 (*byr4(7A) mob1*-R4) was generated by homologous recombination (Keeney and Boeke, 1994) of KGY7401 (*byr4::ura4*$^+$ *mob1*-R4; Salimova *et al.*, 2000) with *Xho*I- and *Pst*I-digested plasmid pKG5876. The resulting DNA fragment contained the mutated *byr4* open reading frame as well as 595 and 373 base pairs of its 5′ and 3′ untranslated regions, respectively. Colonies were selected on YE medium supplemented with 1.5 mg/ml 5-fluoroorotic Acid (5-FOA), and gene replacement was confirmed by whole-cell PCR with primers outside of the integration cassette, as well as by sequencing. The *mob1*-R4 allele was outcrossed with *mob1* to generate strain KGY15846 (*byr4(7A):ura*).

Plasmid construction

Plasmid pKG5876 was generated by site-directed mutagenesis of the pSK plasmid containing a genomic fragment of the *byr4* locus including 595 and 373 base pairs of its 5′ and 3′ untranslated regions, respectively.

HBH purification

Cells were grown in 2 l of 4× YE media to mid log phase. Byr4-HBH was purified using a modified version of a two-step tandem affinity purification under fully denatured conditions (Tagwerker *et al.*, 2006). Briefly, cells were lysed by bead disruption into buffer 1 (8 M urea, 300 mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40, and 4 mM imidazole, pH 8.0) and incubated with nickel-nitriloacetic acid (Ni-NTA) agarose beads (Qiagen,Valencia, CA) for 3 h at room temperature. Ni-NTA beads were washed 4× with buffer 3 (8 M urea, 300 mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40, and 20 mM imidazole, pH 6.3) and eluted with buffer 4 (8 M urea, 200 mM NaCl, 50 mM $NaPO_4$, 0.5% NP-40, 2% SDS, 100 mM Tris, and 10 mM EDTA, pH 4.3). The eluate was adjusted to pH 8 before incubating with Streptavidin UltraLink Resin (Pierce, Rockford, IL) overnight at room temperature. Streptavidin beads were washed 4× with buffer 6 (8 M urea, 200 mM NaCl, 2% SDS, and 100 mM Tris, pH 8.0) and 1× with buffer 7 (8 M urea, 200 mM NaCl and 100 mM Tris, pH 8.0). Purified Byr4-HBH protein was digested off the streptavidin beads for MS analysis.

2D-LC MS/MS Analysis

Purified Byr4-HBH on streptavidin beads was washed three times with Tris-urea buffer (100 mM Tris, pH 8.0, 8 M urea). Proteins were reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and digested with trypsin, chymotrypsin, elastase, or GluC (Promega, Madison, WI). Purified recombinant MBP-Byr4 phosphorylated by Cdk1 was resolved by SDS–PAGE, followed by Coomassie blue staining. Protein bands were excised, and proteins were reduced, alkylated, and in-gel digested with trypsin or chymotrypsin, respectively. MS analysis was performed with an HPLC (nanoAcquity; Waters, Milford, MA) coupled LTQ mass spectrometer (Thermo Scientific; West Palm Beach, FL). A gradient of 0–40% acetonitrile for 60 min was used. One full precursor MS scan (400–2000 mass-to-charge ratio), and four tandem MS scans of the most abundant ions detected in the precursor MS scan under dynamic exclusion were performed. Ions with a neutral loss of 98 Da (singly charged), 49 Da (doubly charged), or 32.7 Da (triply charged) from the parent ions during the tandem MS scans (MS^2) was further subjected to MS/MS fragmentation (MS^3). MS data analysis was done as previously described (Chen *et al.*, 2013), with the following changes. A newer version of Scaffold (Scaffold_4.3.2) was used, and the filtering criteria were changed to minimum of 90.0% peptide identification probability, minimum of 99.9% protein identification probability, and minimum of four unique peptides. A newer version of Scaffold PTM (version 2.1.3) was also used for phosphorylation analysis. The identified phosphorylation sites were considered confident with an Ascore of at least 19.0 (Beausoleil *et al.*, 2006).

In vitro kinase assays

MBP and MBP-Byr4 fusion proteins were expressed from the pMAL-c2 vector in *Escherichia coli* BL21-CodonPlus-RIL cells by induction with 0.4 mM isopropyl β-D-1-thiogalactopyranoside overnight at 18°C. Recombinant proteins were purified on amylose beads (NEB, Ipswich, MA) in binding buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT) and eluted with excess amounts of maltose. Protein amounts were assessed by Coomassie blue staining, and 25 ng of recombinant Byr4 protein was used as substrate in each kinase reaction. Kinase reactions were performed in protein kinase buffer (10 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT) with 10 µM cold ATP, 3 µCi of [^{32}P]ATP, and 100 ng of kinase-active or kinase-dead insect cell–produced Cdc2-Cdc13 at 30°C for 30 min. Reactions were quenched by the addition of SDS sample buffer. Proteins were separated by SDS–PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and phosphorylated proteins were visualized by autoradiography.

S. pombe protein methods

Cells were lysed by bead disruption in NP-40 buffer containing cOmplete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), 100 mM phenylmethylsulfonyl fluoride, and 0.5 M benzamidine. Proteins were immunoprecipitated with 2 µg of Byr4 antiserum raised against full-length protein (Cocalico Biologicals, Reamstown, PA) under denatured conditions (NP-40 containing SDS; Gould *et al.*, 1991). For gel mobility shifts, immunoprecipitates were washed in phosphatase buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–NaOH, pH 7.4, and 150 mM NaCl) and incubated at 30°C for 30 min with or without λ-phosphatase (NEB). Proteins were separated on 6% Tris-glycine gels containing 2 µM Phos-tag and 40 µM $MnCl_2$ (Kinoshita *et al.*, 2009) and transferred to PVDF membranes by electroblotting. After incubation of membranes with polyclonal rabbit anti-Byr4 (1:5000; Cocalico) or monoclonal mouse anti-PSTAIRE (Sigma-Aldrich, St. Louis, MO) antibodies and subsequent incubation with IRDye 800CW goat anti-rabbit or goat anti-mouse secondary antibodies (LI-COR, Lincoln, NE), proteins were visualized by an Odyssey infrared imaging system (LI-COR).

Indirect immunofluorescence

Cells were grown to early log phase and fixed with ice-cold methanol for at least 10 min at –20°C. For protoplasting, cells were incubated with lysing enzymes (0.3 mg/ml Zymolyase 20T and 0.5 mg/ml lysing enzyme). Cells were washed in phosphate-buffered saline (PBS) and incubated in PBAL (PBS + 1% bovine serum albumin and 100 mM lysine) for 7 h while rotating, followed by an incubation of at least 16 h with Byr4 antiserum (1:200; Cocalico). Cells were washed and incubated with Alexa Fluor 488 goat anti-rabbit immunoglobulin G antibody (1:100) for 6 h to detect Byr4.

Microscopy methods

Microscopy images were obtained with a personal DeltaVision System equipped with an Olympus IX71 microscope using a 100×/numerical aperture 1.40 UplansApo oil immersion objective. Images were processed using a CoolSnap HQ^2 camera and Softworx software. To visualize nuclei and septa, cells were fixed in 70% EtOH at –20°C for at least 15 min before washing with PBS, staining with methyl blue for 30 min, and mounting on a microscope slide with

4′,6-diamidino-2-phenylindole (DAPI). Images were obtained in *z*-series of 0.45-µm optical sections over 5 µm, deconvolved, and combined into maximum projections. For live-cell differential interference contrast (DIC) and fluorescence movies, the ONIX microfluidics perfusion system was used (CellASIC, Hayward, CA). Cells were loaded into Y04C plates for 5 s at 8 psi and YE liquid media flowed continuously into the chamber at 5 psi during imaging. For DIC movies, single images were taken every 20 min. For fluorescence movies, *z*-series were acquired in 0.45-µm optical sections over 5 µm every 5 min. Resulting images were deconvolved and subsequently combined into maximum projections. Overlays and analyses of images and movies were performed using ImageJ software (Schneider *et al.*, 2012).

Spot growth assays

Yeast cell cultures were grown to mid log phase, adjusted to an OD_{600} of 0.1, and serial 10-fold diluted up to 1:10,000. A 4-µl amount of each dilution was spotted onto YE plates, and plates were incubated at the indicated temperatures for 2–3 d.

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